DNA ANALYSIS OF PLEXOR HY DATA

A. SCOPE

Plexor HY data can only be viewed using the Plexor Analysis Software. The Plexor Analysis Software and instructions for software installation can be downloaded from the Promega Web site at: www.promega.com/plexorhy/. Refer to the Plexor HY technical manual for instructions on the initial assay set up parameters.

B. QUALITY CONTROL

B.1 Not applicable

C. SAFETY

C.1 Not applicable

D. REAGENTS, STANDARDS, AND CONTROLS

D.1 Not applicable

E. EQUIPMENT & SUPPLIES

E.1 Equipment

E.1.1 AB 7500 Real-Time PCR instrument and software
E.1.2 Computer
E.1.3 Plexor Analysis Software

E.2 Supplies

E.2.1 Not applicable

F. PROCEDURE

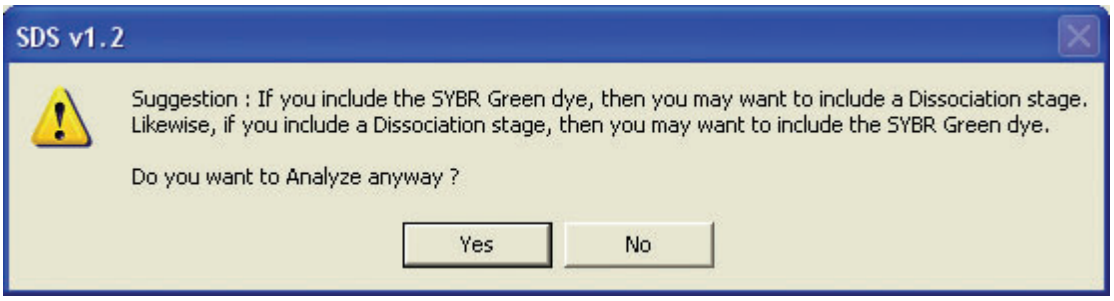
Before data can be analyzed using the Plexor Analysis Software, the raw data must be analyzed in the SDS software, then exported. Two files must be exported: one with amplification data and one with melt / dissociation data.

F.1 Go to the Analysis menu in the SDS software, and select "Analyze" or select the green arrow icon if it is still active. The raw data must be analyzed using the SDS software prior to export.

F.2 To export the amplification data, open the SDS software and select "File", "Export", then "Delta Rn". Save this .csv file with an appropriate name containing the word "amp".

F.3 To export the melt/dissociation data, select "File", "Export", "Dissociation", then "Raw and Derivative Data". Save this .csv file with an appropriate name containing the word "melt."

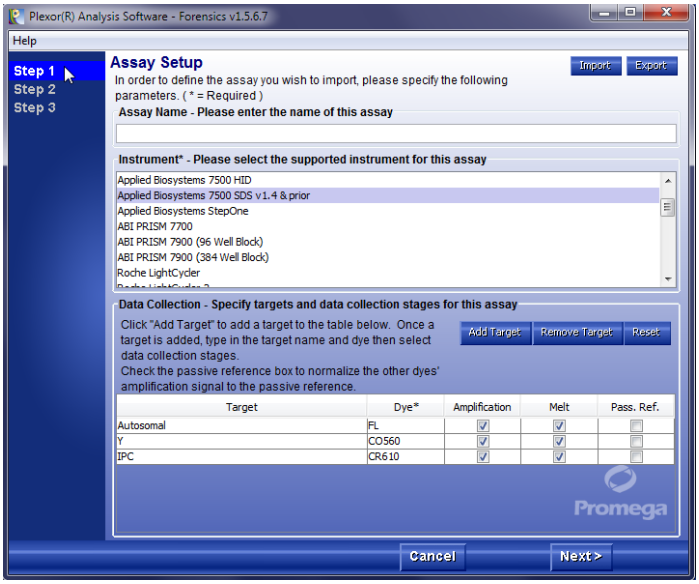
When a dissociation curve is included in a thermal cycling program, the SDS software may expect SYBR green as the dye choice (this is SDS software version dependent). A message will appear when analyzing an experiment. Click "Yes".



F.4 Launch the Plexor Analysis Software.

F.5 Go to the File menu, select "Import New Run", or select the icon: 

F.6 Click "Next" on the Assay Setup window once the initial set up has been performed and the below parameters are selected.



F.7 Click "Next" on the Run Info Screen. The operator name must be inserted; filling out the other run details is optional.

F.8 Use the File Import screen to specify the data files exported from the AB 7500 instrument. Use "Browse" to locate the appropriate exported amplification and

dissociation data files.

F.9 Select "Finish" completing the data import and opening the Analysis Desktop.

F.10 After data import is complete the PCR Curves tab of the Analysis Desktop is displayed.

F.11 Define the DNA standards:

F.11.1 Use the well selector to highlight wells that contain DNA standards.

F.11.2 Select the Create Dilution Series icon.

F.11.3 Confirm that the series selected is "Vertical Series", the series is "Decreasing", 50.0 is entered for the starting concentration, and 5.0 is entered for the dilution factor, then select "Apply".

F.11.4 Highlight the first column of standards. Color code this column by clicking the  button.

F.11.5 (OPTIONAL) Highlight the second column of standards. Color code this column using a different color than the first column.

F.11.6 Highlight the wells containing the standards. Select "Create Standard Curve" using the  button. Do this for both the Auto and Y channel.

F.12 Set the expected target melt temperature (Tm) and the expected target melt temperature range:

F.12.1 Select wells containing the DNA standards. The Tm for each selected sample will be displayed in a table to the right of the graph. The expected target melt temperature and associated target melt temperature range for all samples in this dye channel should be set based on the Tm of these standards. In the melt curves window, move the mouse so that the arrow is over the expected target melt temperature line, and drag it to the midpoint of the melt curves. Alternatively, double-click on the line, and enter the desired temperature. Adjust the expected target melt temperature for all three dyes channels (FL, CO560 and CR610). The average expected target melt temperature for each dye channel is as follows:

Autosomal target (FL): ~79–81°C
Y-chromosomal target (CO560): ~81–83°C
IPC (CR610):~79–81°C

Note: The default setting for the expected target melt temperature is 90.0°C and the default target Tm range is +/–1.5°C. Failure to set the range for the expected target melt temperature correctly will cause the results to be incorrectly reported in the graph legend and reports i.e. Tm=No, which will incorrectly indicate that there wasn't enough amplification product to cross the melt threshold. Some

samples may have the IPC Tm value fall outside this range by as much as 2°C. Adjust the lower bounds of the expected melt range to encompass these samples, if desired. Amplification data, in particular the C_q value, is the primary means of analyzing IPC data.

The melt threshold is the level of signal that must be reached for the Plexor Analysis Software to "call" the melt results. Target Tm indicators are included in the table to the right of the amplification and melt curve windows.

F.13 Define the no-template control reactions:

F.13.1 Use the well selector to highlight the wells that contain NTC reactions.

F.13.2 Select the NTC icon: 

F.14 Assign sample names to the unknowns:

F.14.1 Select the Sample IDs tab. Select the well, and enter the desired sample name. Repeat to enter sample names for other wells.

F.14.2 Enter the same sample name for duplicate samples. **The calculated DNA quantities for samples with the same sample name will be averaged in the Forensic report.**

F.14.3 Sample names can be copied from a Microsoft Excel spreadsheet, provided that the cells are arranged in the same array as your samples (e.g. 8 × 12). Highlight the sample names in the spreadsheet, and select "Copy". In the Edit menu of the Plexor Analysis Software, select "Paste Sample IDs from Template" or use the "Control T" shortcut. The layout of the sample names in the spreadsheet must be the same as the layout of the samples within the PCR plate.

F.15 View the standard curves. One subtab shows the autosomal (FL) standard curve and a second subtab displays the Y (CO560) standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope of –3.3 indicates 100% amplification efficiency. The R^2 value measure the closeness of fit between the standard curve regression line and the individual C_q data points of quantification standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. Verify the following ranges:

Autosomal slope target (FL) range: –3.1 to –4.35
Y-chromosomal slope target (CO560) range: –3.0 to –4.05
R^2 value ≥ 0.990

NOTE: Sample wells for the standard curve may be changed from a "standard" to "unknown" to obtain a slope and R^2 value within the tolerance ranges. The software will automatically incorporate this change into the standard curves. Low R^2 values (R^2 ≤ 0.98) may be due to variability in the amplification results for the replicate samples

of the 0.0032 ng/µL dilution. If necessary, both 0.0032 ng/ µL points may be deleted from the curve. All other concentrations should be represented by at least one datapoint.

F.16 Save the analysis file with the appropriate run ID and then print the autosomal and Y chromosomal standard curves for inclusion in your case notes.

F.17 In the autosomal channel (FL tab) of the PCR Curves, select all samples and DNA standards.

F.18 Select "Create Standard Curve"  to determine the DNA concentrations of the unknowns based on the standard curve. Select "OK" when the software asks if you want to replace the previously created standard curve.

F.19 Repeat Step F.17 and F.18 for the Y channel (CO560 tab). Select "OK" when the software asks if you want to replace the previously created standard curve.

F.20 View the DNA concentrations for all samples in the table next to the standard curve graph. The calculated concentrations can also be viewed in the sample details report or forensic report. The forensic report can be opened by clicking on Forensics / Set Normalization and IPC parameters / OK. This report may be copied into Microsoft Excel and printed.

F.21 Check IPC. The IPC is amplified and detected in the CR610 dye channel under PCR Curves. If the IPC C_q value of an unknown is several cycles higher than that of DNA standards with similar total DNA amounts, inhibition may have occurred and the quantitation data should be interpreted with caution. The samples may be diluted and re-quantitated, or multiple concentrations targeted during amplification. High levels of total human DNA (≥ 10 ng/µL) can cause a slight delay in the IPC crossing the cycle threshold (1–2 cycles).

G. INTERPRETATION GUIDELINES

Definitions used in the Plexor Analysis Software:

[Auto]: Concentration of total human autosomal DNA in a sample in ng/µL (or pg/µL if DNA concentrations were entered as pg/µL).

[Y]: Concentration of human male DNA in a sample in ng/µL (or pg/µL if DNA concentrations were entered as pg/µL).

[Auto]/[Y]: Ratio of total human autosomal DNA concentration to male (Y) DNA concentration. A very high ratio is indicative of a "male/female" mixture with minimal male DNA. If the male contributor is of interest, samples with a very high ratio may benefit from Y STR analysis in addition to, or instead of, autosomal STR analysis. Based on our validation studies, an [Auto]/[Y] ratio of >30 may require Y STR analysis in order to provide the most useful male

information. An [Auto]/[Y] ratio of <10 may be indicative that autosomal STR analysis may be sufficient to obtain maximum information. A ratio greater than 10 and less than 30 may indicate the need to perform both autosomal and Y STR analysis, if possible. It should be noted that one would not expect to obtain any male DNA results using autosomal STR analysis with a ratio of greater than 200. Therefore, the [Auto]/[Y] ratio along with the case synopsis, availability of male reference samples, sample volume, and sample concentration will determine whether autosomal STR analysis or Y STR analysis or both will be utilized.

Note: Due to some individual-to-individual variation in copy number of the autosomal target and Y-chromosomal target, a single-source male DNA sample may not have an [Auto]/[Y] value of 1.0. Values in the range of 0.4 to 2 are commonly observed in single-source male samples.

Curves Status: "OK" indicates the following:
• The sample, if defined as a standard, shows amplification.
• The sample, if defined as a no-template control, shows no amplification.
• A melt peak is present and the Tm is within the expected range.

"Check STD", "Check NTC" or "Check Melts" will be displayed if these criteria are not met.

Notes: It is acceptable for the 0.0032 ng/µL DNA standard to display "No" or "No Call" in the "Tm?" column. Verify that if any subthreshold peak is present in the melt curve, this peak is within the expected target melt temperature range.

The melt threshold is the level of signal that must be reached for the Plexor Analysis Software to "call" the melt results. Target Tm indicators are included in the table to the right of the amplification and melt curve windows. A "Yes" or "No" in the "Tm?" column indicates whether a sample Tm is within the expected target melt temperature range. A "No Call" in this column indicates that the melt curve displays the expected target melt temperature, but there is insufficient amplification product to cause the melt curve to cross the melt threshold.

The "Tm#" is the number of peaks that cross the melt threshold line. More than one peak indicates heterogeneous amplification products. This may be due to nonspecific amplification.

H. REFERENCES

H.1 www.promega.com/plexorhy/

H.2 Plexor HY System for the Applied Biosystems AB 7500 and AB 7500 FAST Real-Time PCR Systems, Instructions for use of products DC1000 and DC1001